

SHORT REPORT

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Two novel CMY-2-type β -lactamases encountered in clinical *Escherichia coli* isolates

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Abstract

Background: Chromosomally encoded AmpC β -lactamases may be acquired by transmissible plasmids which consequently can disseminate into bacteria lacking or poorly expressing a chromosomal *bla*_{AmpC} gene. Nowadays, these plasmid-mediated AmpC β -lactamases are found in different bacterial species, namely *Enterobacteriaceae*, which typically do not express these types of β -lactamase such as *Klebsiella* spp. or *Escherichia coli*. This study was performed to characterize two *E. coli* isolates collected in two different Portuguese hospitals, both carrying a novel CMY-2-type β -lactamase-encoding gene.

Findings: Both isolates, INSRA1169 and INSRA3413, and their respective transformants, were non-susceptible to amoxicillin, amoxicillin plus clavulanic acid, cephalothin, cefoxitin, ceftazidime and cefotaxime, but susceptible to cefepime and imipenem, and presented evidence of synergy between cloxacilin and cefoxitin and/or ceftazidime. The genetic characterization of both isolates revealed the presence of *bla*_{CMY-46} and *bla*_{CMY-50} genes, respectively, and the following three resistance-encoding regions: a *Citrobacter freundii* chromosome-type structure encompassing a *blc-sugE-bla*_{CMY-2-type}-*ampR* platform; a *sul1*-type class 1 integron with two antibiotic resistance gene cassettes (*dfrA1* and *aadA1*); and a truncated mercury resistance operon.

Conclusions: This study describes two new *bla*_{CMY-2-type} genes in *E. coli* isolates, located within a *C. freundii*-derived fragment, which may suggest their mobilization through mobile genetic elements. The presence of the three different resistance regions in these isolates, with diverse genetic determinants of resistance and mobile elements, may further contribute to the emergence and spread of these genes, both at a chromosomal or/and plasmid level.

Keywords: β -lactamase, Resistance regions, Genetic environment, *Escherichia coli*

Background

AmpC β -lactamases, along with Class A β -lactamases, are a major group of clinically important enzymes [1,2]. They belong to class C according to the Ambler classification and to group 1 following the functional classification of Bush-Jacoby [3,4], whose prevalence is increasing worldwide [1]; these β -lactamases are associated with infections caused by pathogenic Gram-negative bacteria, particularly *Escherichia coli* and *Klebsiella pneumoniae*. The identification of isolates containing plasmid-mediated AmpC- β -lactamase (PMA β) is epidemiologically and clinically relevant due to the limitations of treatment options [5].

AmpC enzymes hydrolyse amino- and ureidopenicillins, and cephamycins (cefoxitin and cefotetan) and, at a low level, oxyiminocephalosporins (ceftazidime, cefotaxime, and ceftriaxone) and aztreonam: they are not inhibited by β -lactamase inhibitors such as clavulanic acid [1]. AmpC-producing isolates are susceptible to carbapenems and to zwitterionic cephalosporins (cefepime and cefpirome).

In this study, we performed the phenotypic and molecular characterization of two new CMY-2-types (designated CMY-46 and CMY-50), both encoded by probably chromosomal inducible *ampC* genes, produced by two clinical *E. coli* isolates. The genetic environment of *bla*_{CMY-46} and *bla*_{CMY-50} was also investigated.

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Methods

Bacterial isolate collection

Two clinical *E. coli* strains (INSRA1169 and INSRA3413) were isolated, in 1999, from urine samples of two patients of 77 years and 7 months old, in two different hospitals in Portugal. *E. coli* DH5 α (pBK-CMY-2) strain was used as control for antimicrobial susceptibility tests.

Antimicrobial susceptibility tests

Minimal inhibitory concentrations were determined by a microdilution method according to guidelines of the French Society of Microbiology (SFM 2013, <http://www.sfm-microbiologie.org/>) against seven β -lactams, alone or in combination with clavulanic acid, and against ciprofloxacin, gentamicin and trimethoprim. Isolates non-susceptible to one third-generation cephalosporin, cefoxitin and/or exhibiting synergy with boronic acid and/or cloxacillin, were considered as presumptive AmpC producers. Imipenem and clavulanic acid were used in order to identify induction effect of AmpC [1,6]. Disks of inducing agents (imipenem 10 μ g and amoxicillin plus clavulanic acid 25 + 10 μ g) and disks of cephalosporins (cefotaxime 30 μ g and ceftazidime 30 μ g) were placed on Mueller–Hinton agar plates, 20 mm apart. Positive induction was demonstrated by the antagonism effect surrounding the cephalosporin disks adjacent to the inducers.

Isoelectric point determination

β -Lactamases were characterized by isoelectric focusing of ultrasonicated bacterial extracts with the control strains expressing pI 5.2, 5.6, 7.6, 9.0, 9.2, as previously described [7].

Molecular characterization of *ampC* and ESBL-encoding genes

The presence of acquired *ampC* (*bla*_{CMY}, *bla*_{MOX}, *bla*_{FOX}, *bla*_{LAT}, *bla*_{ACT}, *bla*_{MIR}, *bla*_{DHA}, *bla*_{MOR}, *bla*_{ACC}) and *bla*_{ESBL} genes (*bla*_{TEM}, *bla*_{SHV}, *bla*_{OXA-1-type}, *bla*_{CTX-M}) was investigated by multiplex PCR assays with primers and conditions as described elsewhere [7–10], and those from Table 1. Controls were included in all assays.

Gene transfer experiments

Transferability of the *bla*_{CMY} genes was attempted by both broth mating-out assays and electroporation. Conjugation experiments were performed at 37°C, using recipient strains *E. coli* C600 Rif^R, Str^R and *E. coli* J53 NaN₃^R, according to the antibiotic susceptibilities of the clinical isolates used as donor. Transconjugants were selected on MacConkey agar plates containing 250 μ g/ml of rifampicin, 160 μ g/ml of streptomycin or 160 μ g/ml of sodium azide plus 10 μ g/ml of cefoxitin. Plasmid DNA was extracted from clinical strains, using the Wizard Plus Midipreps DNA Purification kit (Promega), and used to

transform electrocompetent *E. coli* DH5 α Δ *ampC* by electroporation, as previously described [7]. Transformants were selected on Luria broth medium containing 10 μ g/ml of cefoxitin.

Cloning experiments

The *bla*_{CMY-2}, *bla*_{CMY-46} and *bla*_{CMY-50} genes were amplified with iProofTM High-Fidelity DNA Polymerase (Bio-Rad, Hercules, CA), using primers from Table 1. Amplicons (1169 bp) were ligated in the *Sma*I site of the phagemid pBK-CMV (Stratagene) downstream of its inducible *lacZ* promoter and transformed into electrocompetent *E. coli* DH5 α Δ *ampC* cells. A gene Pulser II apparatus (Bio-Rad, Hercules, CA) was used for standard electroporation techniques, as previously described [7]. Recombinant bacteria were selected on LB agar plates containing 10 μ g/ml of cefoxitin.

Genetic background characterization

The presence of class 1 integrons was determined in both isolates through PCR amplification of the integrase-specific *intI1* gene with the same specific primers and conditions as reported previously [11] (Table 1). PCR-mapping and sequencing of the genetic environment of *bla*_{CMY-46} and *bla*_{CMY-50} was performed using primers targeting genes known for promoting antibiotic resistance and integrons (Table 1). Sequence alignments and generation of resistance cassette contigs were performed using *Bionumerics* (Applied Maths). Gene identity was confirmed at the NCBI website (<http://www.ncbi.nlm.nih.gov/>).

Findings

The two clinical *E. coli* isolates INSRA1169 and INSRA3413 were resistant to amoxicillin, amoxicillin plus clavulanic acid, cephalothin, cefoxitin, ceftazidime, cefotaxime, gentamicin and trimethoprim, but susceptible to cefepime and imipenem (Table 2). INSRA1169 was also nonsusceptible to ciprofloxacin. Synergy between cloxacillin and cefoxitin plus cefotaxime and boronic acid, along with the absence of synergy between extended-spectrum cephalosporins and clavulanic acid, suggest that the resistance to extended-spectrum cephalosporins was mediated by the overproduction of AmpC β -lactamases. The resistance phenotype was not transferable, neither in conjugation assays with *E. coli* C600 as a recipient, or in transformation assays by electroporation of plasmid-DNA preparations into *E. coli* DH5 α . This might suggest a chromosomal location of AmpC-encoding genes.

Transformants, obtained after cloning of DNA amplicons of INSRA1169 and INSRA3413 in pBK-CMV plasmid vector (Figure 1a), showed a resistance phenotype similar to that of clinical isolates. However, they were susceptible to ciprofloxacin, gentamicin and trimethoprim, like the control strain *E. coli* DH5 α (pBK-CMY-2).

Table 1 Primers, drawn in this study, used for PCR amplification and sequencing of PMA β genes and for PCR mapping of *bla*_{CMY-46} and *bla*_{CMY-50}

Gene (s)	Primer Sequence (5' → 3')		PCR product (bp) / Method ^a
	Forward	Reverse	
<i>bla</i> _{CMY-G2}	TTACGGAAGTATTTCATG	TCGTGAGTTATTGCAGC	1169 / PCR + Seq.
<i>orf513</i>	GCCAGGTCTTGAGTATCGTC	CATGTAATTGAGTCAGCGTATC	363 / PCR + Seq.
<i>fdrB</i> - <i>fdrD</i>	CTCAGTTGACCACCACGAAC	GAATGCCAATAGCCGTTACGAC	920 / PCR + Seq.
<i>fdrB</i> - <i>ampR</i>	CTCAGTTGACCACCACGAAC	CACCACTCAGAATGTTACGCA	1140 / PCR + Seq.
<i>ampR</i> - <i>bla</i> _{CMY-G2}	TGCGTGAACATTCTGACTGGTG	TTTCTCTGAACGTGGCTGGC	1660 / PCR + Seq.
<i>bla</i> _{CMY-G2} - <i>sugE</i>	TGGCCAGAACTGACAGGCAAA	ATGTCCTGGATCGTTTTATTA	1751 / PCR + Seq.
<i>merA</i> - <i>urf2</i>	TTCCCTACCTGACGATGG	TGTTGCAGGCAGGAATAGC	1214 / PCR + Seq.
<i>merR</i> - <i>merA</i>	TCITTCTCCCTTGACGCG	CACCTTGTGCAACAGCCCA	Variable / PCR + Seq.
<i>merA</i>	CGTCCAATCTGCCATAGTG	GTAGGGGAACAACCTGGTCG	Seq.
<i>merD</i>	CCTTCGAGCGGGTATC	CCGATACCCGCTCGAAG	Seq.

^aMethod used for screening and/or identification of genes: M-PCR, Multiplex-PCR; Seq, sequencing; PCR + Seq, PCR and sequencing.

(Table 2). Both the clinical strains and the transformants produced β -lactamases exhibiting an alkaline isoelectric point (pI 9.2) compatible with AmpC-type β -lactamases. Indeed, PCR revealed the absence of *bla*_{ESBL} genes plus the presence of chromosomal *E. coli ampC*-type gene; the sequence of cloned DNA fragments identified two new CMY-2-like genes, which were not related to chromosome-mediated *E. coli* AmpC gene. The chromosomal location of such genes has only been observed in *Salmonella* spp. and *Proteus mirabilis* isolates [12–14].

The deduced amino acid sequences confirmed that the new genes encoding the β -lactamases CMY-46 (in INSRA1169) and CMY-50 (in INSRA3413), which were new variants of CMY-2, differed by 9 and 13 amino acid substitutions, respectively (Table 3). Two of these mutations (Q193K plus P208A for CMY-46 and N194S plus D198N for CMY-50) are in the Ω loop (between amino acids 178 and 226), which interacts by hydrogen bonding with helix H-2 close to the active Ser64. Substitutions in this region have been linked to the extension of the hydrolysis spectrum [15]. However, CMY-46 and

CMY-50 β -lactamases did not confer resistance to cefepime and conferred low level of resistance to ceftazidime and cefotaxime, which suggests that, in contrast to extended-spectrum AmpCs, they have moderate or no extended-spectrum activity (Table 2) [15–18].

The study of sequences surrounding *bla*_{CMY-46} and *bla*_{CMY-50} revealed the presence of the *blc* gene (encoding an outer membrane lipoprotein) and the *sugE* gene (encoding a small MDR protein responsible for resistance to quaternary ammonium compounds) downstream of their open reading frames (Figure 1a). Upstream, an *ampR* gene encoding the usual transcriptional regulator of *ampC* genes was observed in an opposite direction of transcription (Figure 1a). The presence of an intact *ampC-ampR* segment in both new *bla*_{CMY} genetic regions implied that the production of CMY-46 and CMY-50 is inducible, which was corroborated by the used phenotypic induction method. This *ampC-ampR* region was identical to the sequence flanking the *bla*_{AmpC} gene in the *C. freundii* chromosome [6], except for *AmpR*_{CMY-46} (that had 4 amino acid substitutions), but none were located in the helix-turn-helix region or in other

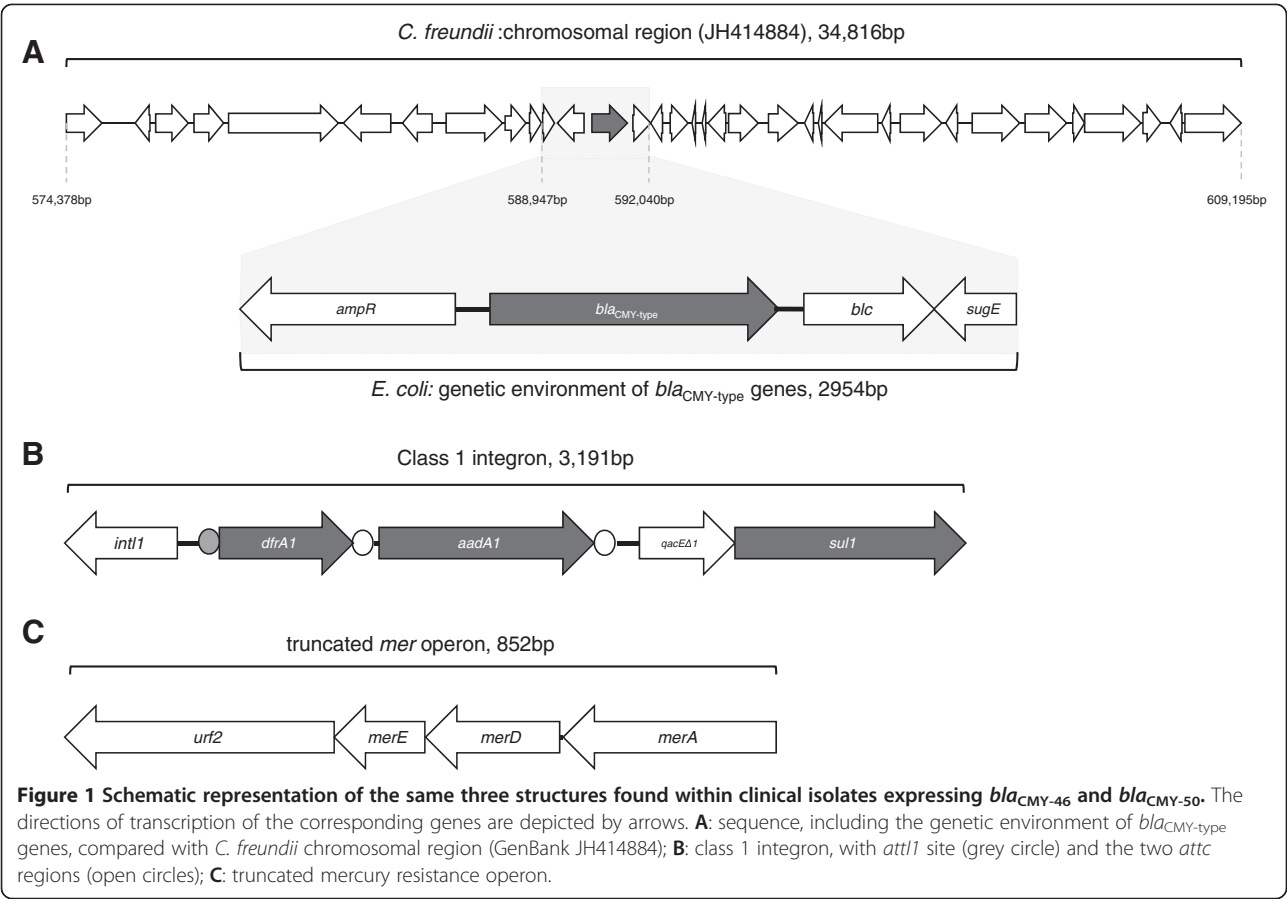
Table 2 MICs of antibiotics for CMY-46- and CMY-50-producing *E. coli* isolates and *E. coli* transformants and recipients^a

<i>E. coli</i> strain	MIC (μ g/ml) ^b											
	AMX	AMC ^c	CF	CAZ	CCAZ ^c	CTX	FEP	FOX	IMP	CIP	GEN	TMP
DH5 α Δ <i>ampC</i>	8	8	8	0.25	0.125	0.06	0.03	4	0.25	≤0.125	≤0.125	≤0.25
DH5 α (pBK-CMY-2)	>2048	>2048	1024	32	16	8	0.25	64	0.5	≤0.125	1	≤0.25
INSRA1169 (CMY-46 + TEM-1)	>2048	>2048	1024	16	4	4	0.032	64	2	4	>128	>128
DH5 α (pBK-CMY-46)	>2048	>2048	1024	32	4	8	0.25	64	0.5	≤0.125	2	≤0.25
INSRA3413 (CMY-50)	>2048	>2048	1024	16	8	4	0.25	64	2	≤0.125	64	64
DH5 α (pBK-CMY-50)	>2048	>2048	1024	16	8	4	0.25	64	0.5	≤0.125	0.5	≤0.25

^a*E. coli* EcDH5 α (pBK-CMY-2) was control strain; *E. coli* DH5 α (pBK-CMY-46) and *E. coli* DH5 α (pBK-CMY-50) were transformants of *E. coli* INSRA1169 (harboring CMY-46 and TEM-1 enzymes) and *E. coli* INSRA3413 (harboring CMY-50 enzyme), respectively; *E. coli* EcDH5 α was the recipient strain.

^bAMX, amoxicillin; AMC, amoxicillin-clavulanic acid; CF, cephalothin; CAZ, ceftazidime; CCAZ, ceftazidime-clavulanic acid; CTX, cefotaxime; FEP, cefepime; FOX, cefoxitin; IMP, imipenem; CIP, ciprofloxacin; GEN, gentamicin and TMP, trimethoprim.

^cClavulanic acid, at fixed concentration of 2 μ g/ml.



positions related to AmpR function [20,21]. The promoter regions of our *bla*_{CMY-2-type} and *ampR* genes harbored no sequence element associated with increased strength of the promoter [20,21]. In addition, the *frdD*, *frdC*, and *frdB* genes that are usually adjacent to *ampC-ampR* in the *C. freundii* chromosome were not identified in the sequences flanking *bla*_{CMY-46} or *bla*_{CMY-50}.

Class 1 integrons, also detected in INSRA1169 and INSRA3413 (Figure 1b), comprised the integrase-encoding gene *intI1*, two gene cassettes, *aacA1* and *dfrA1*, and *qacEA1* plus *sul1*, which were probably responsible for the observed resistances to trimethoprim and aminoglycosides.

We also found a truncated mercury resistance operon (Figure 1c), which was previously reported as belonging to a “kan region” that included a kanamycin resistance gene [22]. This finding is of concern since mercury resistance may help to promote antibiotic resistance through indirect selection [23].

In summary, this study describes two new *bla*_{CMY-2-type} genes located within a *C. freundii*-derived fragment. Considering that CMY-type β-lactamases, detected in *E. coli*, are derived from the *C. freundii* chromosomal AmpC [1] and that chromosome-derived genes are usually mobilized by MGE [24,25], the presence of three

Table 3 Comparison of amino acid substitutions of two new CMY-type β-lactamases

PMAβ	Amino acid at position no. ^a																			pI	Accession Number
	3	3	4	1	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	3	
	2	5	9	0	0	2	2	3	4	6	9	9	9	9	0	3	4	5	6	4	
				2	5	4	6	3	3	4	0	3	4	8	8	6	2	3	1	8	
CMY-2	V	Q	A	Q	R	D	R	H	T	K	T	Q	N	D	P	A	H	A	R	V	9.0 X91840
CMY-46			T		S		T	R	A			K			A	V	R				9.2 FN556186
CMY-50	I	E		R	S	E	T	R		Q			S	N				E	C	A	9.2 FN645444

^aNumbering according to Bauernfeind et al., 1996 [19].

resistance regions with diverse resistance determinants and MGE in this study, suggests the dynamics of bacteria in the transference of antibiotic resistance. In addition, they might also trigger the future emergence and spread of these resistant determinants both at a chromosomal or/and plasmid level.

Availability of supporting data

The data set supporting the results of this article is included within the article.

Abbreviations

PMAβ: Plasmid-mediated AmpC-β-lactamase; SFM: French Society of Microbiology; MDR: Multidrug-resistance; MGE: Mobile genetic elements; ESAC: Extended-spectrum AmpC cephalosporinases; ESBL: Extended-spectrum β-lactamase.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

VM performed the experiments, data interpretation and drafted the manuscript; EF and MF took part in the experiments; MP and FF worked on microbiology and clinical data; RB participated in data interpretation and reviewing of the manuscript; and MC conceived the study, and contributed to data interpretation and reviewing of the manuscript. All authors read and approved the final manuscript.

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